



Jowiya, W., Brunner, K., Abouelhadid, S., Hussain, H. A., Nair, S. P., Sadiq, S., Williams, L. K., Trantham, E. K., Stephenson, H., Wren, B. W., Bajaj-Elliott, M., Cogan, T. A., Laws, A. P., Wade, J., Dorrell, N., & Allan, E. (2015). Pancreatic Amylase is an Environmental Signal for Regulation of Biofilm Formation and Host Interaction in *Campylobacter jejuni*. *Infection and Immunity*, 83(12), 4884-4895. <https://doi.org/10.1128/IAI.01064-15>

Peer reviewed version

Link to published version (if available):  
[10.1128/IAI.01064-15](https://doi.org/10.1128/IAI.01064-15)

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**Pancreatic Amylase is an Environmental Signal for Regulation of Biofilm  
Formation and Host Interaction in *Campylobacter jejuni***

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Running head: *C. jejuni* response to pancreatic amylase

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## 21        **Abstract**

22        *Campylobacter jejuni* is a commensal bacterium in the intestines of animals and  
23        birds and a major cause of foodborne gastroenteritis in humans worldwide. Here we  
24        show that exposure to pancreatic amylase leads to secretion of an  $\alpha$ -dextran by *C.*  
25        *jejuni* and that a secreted protease, Cj0511, is required. Exposure of *C. jejuni* to  
26        pancreatic amylase promotes biofilm formation *in vitro*, increases interaction with  
27        human epithelial cell lines, increases virulence in the *Galleria mellonella* infection  
28        model and promotes colonisation of the chicken ileum. We also show that exposure  
29        to pancreatic amylase protects *C. jejuni* from stress conditions *in vitro* suggesting  
30        that the induced  $\alpha$ -dextran may be important during transmission between hosts.  
31        This is the first evidence that pancreatic amylase functions as an inter-kingdom  
32        signal in an enteric micro-organism.

33

## 34        **Introduction**

35        *Campylobacter* infection is a major cause of foodborne gastroenteritis in humans  
36        worldwide (1). Disease ranges from mild, non-inflammatory, self-limiting diarrhea to  
37        prolonged, inflammatory diarrhea and occasional serious complications such as  
38        Guillain-Barré syndrome and reactive arthritis (1). Ninety percent of human disease  
39        is attributed to *C. jejuni*, while *Campylobacter coli* accounts for the remainder (2).

40        In contrast to human infection, *C. jejuni* establishes a largely asymptomatic  
41        but persistent infection in animals and birds, and the major risk of human infection is  
42        from handling and consumption of poultry meat (3, 4, 5). Campylobacters primarily  
43        inhabit the lower gastrointestinal (GI) tract of poultry and contamination of the flesh

44 of the bird occurs at slaughter. *Campylobacter* spp. are present in the luminal crypts  
45 of the chicken gut as densely-packed communities surrounded by mucus (6), an  
46 arrangement suggestive of a biofilm, a structured community of bacteria enclosed in  
47 a self-produced exopolymeric matrix (EPM) composed primarily of polysaccharides  
48 (7). Biofilms have also been described on experimentally-infected human ileum ex  
49 vivo suggesting that this mode of growth is also important during human infection (8,  
50 9).

51 Despite several studies of *C. jejuni* biofilms (10-15), the EPM has not been  
52 characterised. Polysaccharide is a major component of most bacterial EPMs and  
53 provides the structural framework (7, 16, 17). *C. jejuni* produces several surface-  
54 associated carbohydrate structures including lipooligosaccharide (LOS), capsular  
55 polysaccharide (CPS) and both *N*- and *O*-linked glycoproteins (18), but there is no  
56 evidence that these glycans contribute to biofilm formation (11, 19).

57 Enteric bacteria use environmental signals present in host environments to  
58 regulate the expression of components required for interaction with the host and  
59 signals include bile salts, mucus, low oxygen, bicarbonate and neuroendocrine  
60 stress hormones (20-22). *C. jejuni* shows increased interaction with intestinal  
61 epithelial cells (IECs) in response to low oxygen, bile salts and noradrenaline (23-  
62 25). In addition, bile salts have recently been reported to enhance *C. jejuni* biofilm  
63 formation by causing release of extracellular DNA (26).

64 Here we show that *C. jejuni* responds to the presence of pancreatic amylase  
65 by secreting an  $\alpha$ -dextran that is a component of the biofilm EPM. This is the first  
66 definitive characterisation of the polysaccharide component of *C. jejuni* biofilms.  
67 Importantly, pre-exposure to pancreatic amylase results in significant changes in the

68 interactions of *C. jejuni* with the host in both *in-vitro* and *in-vivo* infection models.  
69 Pre-exposure to pancreatic amylase also promotes stress tolerance *in vitro*  
70 suggesting that the  $\alpha$ -dextran may be important during transmission. This is the first  
71 evidence that pancreatic amylase is used by an enteric micro-organism as a signal  
72 to regulate interaction with the host.

73

## 74 **MATERIALS AND METHODS**

75 **Bacterial strains and growth conditions.** Bacterial strains are listed in Table 1. *C.*  
76 *jejuni* was stored at -70°C in Brucella broth (BB; Oxoid) containing 15% glycerol and  
77 was grown on Mueller Hinton agar (MHA; Oxoid), Brucella agar (BA; Oxoid),  
78 Columbia blood agar (CBA; Oxoid) containing 7% defibrinated horse blood (E & O  
79 Laboratories) in a microaerobic atmosphere generated using Campypaks (Oxoid) in  
80 gas jars at 37°C. Charcoal (0.4%) was added to BA to improve contrast for  
81 photography. For broth cultures, BB or Eagle's Minimal Essential Medium- $\alpha$  (MEM-  
82  $\alpha$ ; Sigma-Aldrich, M0894) were used and incubated with shaking (50 rpm) at 37°C in  
83 5% CO<sub>2</sub>. Kanamycin (Km; 40  $\mu$ g/ml) and chloramphenicol (Cm; 20  $\mu$ g/ml) were used  
84 for selection.

85

86 **Sources of pancreatic amylase.** Hog pancreatic  $\alpha$ -amylase (HPA) was purchased  
87 from Sigma Aldrich. Polyhistidine-tagged human pancreatic amylase expressed and  
88 purified from human cells was obtained from Sino Biologicals. For chicken pancreas  
89 extract, 20 organs were homogenized in 0.05 M Tris, 0.9% NaCl, 0.05 M CaCl<sub>2</sub>, pH

90 8.0 (27). After centrifugation (1,000 x g, 20 min), amylase activity in the supernatant  
91 was quantified by enzymatic assay (28).

92

93 **EPM purification and characterisation.** Growth (48 h) removed from MHA with or  
94 without 100 nM HPA was suspended in phosphate buffered saline (PBS) and the  
95 extracellular material was recovered using modifications of a method previously used  
96 to isolate exo-polysaccharide from *Vibrio parahaemolyticus* (29). Briefly, the bacterial  
97 cell suspensions in PBS were washed on a rotary platform at 200 rpm for 1.5 h at  
98 30°C, vortexed for 15 min and washed again at 200 rpm for 1.5 h at 20°C. The  
99 supernatant was recovered (1,800 x g, 15 min) and precipitated overnight with 4  
100 volumes of cold acetone at 4°C. The precipitate was recovered (450 x g, 5 min),  
101 washed in distilled water (dH<sub>2</sub>O), dried (Speedvac SPD1010, Thermo Scientific),  
102 dissolved in dH<sub>2</sub>O and stored at -70°C. Total carbohydrate was measured by phenol-  
103 sulphuric acid assay (30) with glucose as standard and statistical significance was  
104 assessed using a two-sample t-test. Further characterisation of the EPM  
105 carbohydrate was performed after dialysis (14 kDa MWCO) for 72 h at 4°C against  
106 three changes of dH<sub>2</sub>O per day. The EPM was freeze dried and characterized using  
107 NMR spectroscopy (31), monomer analysis (32) and linkage analysis (33) in  
108 comparison with a commercial  $\alpha$ -dextran standard (Sigma-Aldrich).

109

110 **Genetic complementation of a *cj0511* mutant.** A *cj0511* mutant obtained from a  
111 collection at the London School of Hygiene and Tropical Medicine (Table 1) was  
112 used to construct a complemented strain by insertion of *cj0511* under the control of

the iron-inducible *fdxA* promoter into a pseudogene (*cj0046*) in the *cj0511* mutant as previously described (34). The oligonucleotides used are listed in Table 2.

#### **Production of recombinant Cj0511 and proteolysis assay**

Codon optimized *cj0511* was synthesized by Celtek, USA and obtained in pGH. The gene was cut by HindIII (New England Biolabs, UK) and the released DNA fragment was cloned in pET-28a(+)(Novagen, UK) to generate pMAR31. Expression of *cj0511* was achieved in *E.coli* BL21 (DE3) (New England Biolabs, UK). To localize the recombinant protein in the periplasm of *E.coli*, recombinant Cj0511 was modified to have a signal peptide from the *E.coli* DsbA protein fused to the N-terminal, while the C-terminus was fused to a 6 x-His affinity tag. Recombinant Cj0511 was purified using nickel affinity column (Qiagen) and eluted in 500 mM imidazole, 300 mM sodium phosphate and 20 mM sodium chloride buffer. The eluted fractions were combined and concentrated using an Amicon ultrafiltration column with a 100 kDa cut off (Merck Millipore, UK) then resuspended in 10 mM sodium phosphate buffer. One microgram of purified r-Cj0511 was mixed with 2 µg polyhistidine-tagged human pancreatic amylase (Sino Biologicals) or casein (Sigma Aldrich) in 20 µL of 50 mM sodium phosphate buffer (pH 7.5) and incubated at 37°C for a total of 2 h. The reactions were stopped using 6 x Laemmli buffer and the reaction mixtures were boiled for 10 min before being separated by SDS-PAGE (16% polyacrylamide) and stained with Coomassie R-250.

**Biofilm assays.** A 16 h culture of *C. jejuni* in BB was diluted to OD<sub>600</sub> of 0.1 in BB with or without 100 nM HPA, 100 nM recombinant human pancreatic amylase or an equivalent activity of chicken pancreas extract, added to a borosilicate glass tube (VWR International) and incubated without shaking at 37°C in 5% CO<sub>2</sub>. After 48 h the culture was decanted, the biofilms were washed with dH<sub>2</sub>O, dried and stained for 5 min with 0.1% (w/v) crystal violet (CV; Sigma-Aldrich), washed with dH<sub>2</sub>O and dried. Bound CV was solubilised in 80% ethanol-20% acetone and quantified by measuring OD<sub>600</sub>. Statistical significance was assessed with a two-sample t-test assuming unequal variance.

**Confocal laser scanning microscopy of biofilm.** A 16 h culture of *C. jejuni* was diluted to OD<sub>600</sub> of 0.1 in BB with or without 100 nM HPA and placed in a 6-well tissue culture plate (Sarstedt). Two glass coverslips were placed upright in each well and incubated for 48 h at 37°C in 5% CO<sub>2</sub>. The coverslips were washed in PBS, stained with Live/Dead BacLight stain (Invitrogen) according to the manufacturer's instructions and visualised with a BioRad Radiance 2100 confocal scanning system attached to an Olympus BX51 upright microscope. Digital images were produced using Image J software (National Institutes of Health).

**Adhesion and invasion of Caco-2 cells.** Human colon cancer cells (Caco-2) were cultured as previously described (25) and 10<sup>8</sup> bacteria, grown on MHA with or without 100 nM HPA, were added at a multiplicity of infection (MOI) of 100:1 and incubated at 37°C in 5% CO<sub>2</sub>. Interacting and invading bacteria were enumerated at



3, 6 and 24 h as previously described (25) and statistical significance was assessed using a two-sample t-test assuming unequal variance.

**Interaction with T84 human colonic epithelial cells.** T84 cells were grown in complete Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Invitrogen) in rat collagen pre-coated transwell dishes until the transepithelial electrical resistance (TEER) measurement (35) was  $>800\ \Omega$  confirming formation of tight-junctions in the monolayer. Co-culture studies were performed at a MOI of 10:1 in DMEM/F12 HAM medium containing 10% foetal bovine serum for 6 or 24 h. Bacteria were grown on MHA with or without 100 nM HPA and viable counts were performed. Statistical significance was assessed using a Wilcoxon matched paired test. IL-8 levels were determined by ELISA (eBioscience) from the supernatant of the apical surface at 24 h post-infection.

***Galleria mellonella* infection model.** *G. mellonella* larvae were obtained commercially (Cornish Crispa Co) and stored in containers with wood chips at 12°C. *C. jejuni* strains were grown on MHA with or without 100 nM HPA for 48 h, harvested in PBS and  $10^6$  cfu injected into the right foreleg of 10 *Galleria* larvae as previously described (36). Statistical significance was assessed using a two-sample t-test, assuming unequal variance.

**Infection of chickens.** Day-old Ross broiler chickens were obtained from a commercial supplier and housed in biosecure housing. The study was performed

under a project licence issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986 (PPL 30/2599) and protocols and euthanasia techniques were approved by the University of Bristol Animal Welfare Ethical Review Board. Birds were fed commercial starter and grower diets (BOCM Pauls Ltd, Ipswich), had access to food and water *ad libitum* and were exposed to a 12 h light/darkness cycle. At 21 days old, groups of 30 birds were infected by oral gavage with  $10^5$  of *C. jejuni* 11168H or the *cj0511* mutant grown on MHA with or without 100  $\mu$ M HPA for 48 h and resuspended in MH broth. Fifteen birds were euthanized by cervical dislocation at 4 and 7 days post infection and ileum and liver samples removed for culture. Ileum samples were serially diluted and plated on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid) incubated at 37°C for 48 h in microaerobic conditions. Liver samples were homogenised and enriched in modified Exeter broth (37), incubated with minimal headspace at 37°C for 48 h and plated on mCCDA. Differences in the number of birds colonised were assessed using a Chi-square test. Differences in the number of bacteria found at each site were analysed using a Kruskal-Wallis test with Dunn's multiple comparison test.

**Resistance to environmental stress.** The growth from 48 h MHA plates with and without 100 nM HPA was recovered into PBS (4°C) or MH broth (20°C), washed and resuspended to an OD<sub>600</sub> of 2.0. The cultures were incubated with shaking (50 rpm) at 37°C in 5% CO<sub>2</sub> and samples were removed at intervals for viable counting. The statistical significance of the difference in viable counts in the presence and absence of HPA was assessed using two sample t-tests, assuming unequal variance.

## RESULTS

### ***C. jejuni* responds to the presence of pancreatic amylase.** Physiological

concentrations of mammalian pancreatic  $\alpha$ -amylase are estimated to be nanomolar (38). When hog pancreatic  $\alpha$ -amylase (HPA) was incorporated into agar at various concentrations, we observed the formation of large mucoid colonies of *C. jejuni* 11168H at a minimum concentration of 100  $\mu$ M (Fig. 1A). It is important to note that mucoid colonies were apparent on MHA and also on BA containing 100  $\mu$ M HPA; since BA does not contain starch, this indicates that the response is independent of amylase starch degrading activity. Whilst 100  $\mu$ M pancreatic amylase seem high compared to the estimated *in vivo* concentrations, it is noteworthy that the number of molecules of amylase that can interact with the bacteria growing on an agar plate is likely to be low because of spatial confinement, in contrast to physiological conditions where bacteria will be bathed in litres of pancreatic juice per day. Mucoid colonies were also produced by other *C. jejuni* strains (81-176, 81116, G1, X) in response to 100  $\mu$ M HPA but not by the periodontal pathogen, *Campylobacter rectus* NCTC 11489 (data not shown).

**Pancreatic amylase promotes *C. jejuni* growth.** As the colonies on amylase were larger in addition to being mucoid, the number of bacteria per colony was determined for *C. jejuni* 11168H from HPA-containing (100  $\mu$ M) and amylase-free MHA. In three experiments, colonies contained a mean of  $5.3 (+/-3.2) \times 10^7$  cfu in the presence of amylase, compared to  $3.3 (+/-2.3) \times 10^6$  cfu in the absence of amylase, representing a 16-fold increase. In MEM- $\alpha$ , supplementation with physiological concentrations (100 nM) of HPA resulted in an increased growth rate (mean generation time of 180 (+/-0) minutes in the presence of pancreatic amylase compared to 280 (+/-35)

minutes in its absence). The difference in growth rate was statistically significant ( $p < 0.001$ ) at both mid-exponential phase (10 h) and stationary phase (15 h) (Fig. 1B).

**Mucoid colonies secrete increased amounts of carbohydrate.** As mucoid colonies are suggestive of exo-polysaccharide production, we attempted to purify the extracellular material from colonies of strain 11168H grown in the presence of increasing concentrations of HPA by washing in PBS. Measurement of the carbohydrate content of this preparation by phenol sulphuric acid assay showed that exposure to physiological concentrations of HPA (100 nM) resulted in a 100% increase in the soluble carbohydrate secreted by *C. jejuni* 11168H compared to growth without HPA (Fig. 2A). A 11168H *kpsM* mutant, unable to export CPS (39), and 11168H *waaF* and *waaC* mutants lacking the outer and entire core oligosaccharide of LOS, respectively (40, 41), also showed increased carbohydrate secretion of comparable magnitude to the wild-type (WT) strain in the presence of HPA, indicating that the secreted carbohydrate is independent of both CPS and LOS (Fig. 2B). The amount of carbohydrate secreted by the 11168H *kpsM*, *waaC* and *waaF* mutants did not differ significantly from the wild-type strain in either the presence or absence of pancreatic amylase.

**The secreted carbohydrate is an  $\alpha$ -dextran.** The soluble extracellular material recovered from *C. jejuni* 11168H and the *kpsM* mutant provided  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra identical to an  $\alpha$ -dextran standard (Fig. 2C) and distinct from starch (Fig. S1). A series of 2D-NMR (COSY, HSQC & HMBC) were also recorded (data not

shown) to confirm the identity of the exo-polysaccharide as an  $\alpha$ -dextran. Monomer analysis was undertaken using high performance anion exchange chromatography in combination with pulsed amperometric detection. After acid hydrolysis, the extracellular material recovered from both *C. jejuni* 11168H and the *kpsM* mutant gave chromatographs containing a single peak which co-eluted with a glucose reference standard (Fig. S2). Monomer analysis thus confirmed that glucose was the only monosaccharide present and linkage analysis showed exclusively 1,6-glycosidic links (Fig. S3).

**Cj0511 is essential for the response to pancreatic amylase but does not degrade it *in vitro*.** Since HPA improves the growth of *C. jejuni*, we reasoned that it could be degraded by the bacterium. As Cj0511 is a protease (42) that is known to be secreted in outer membrane vesicles (OMVs) (43) and is abundant in a *C. jejuni* strain that colonises chickens efficiently (44), we hypothesized that it is involved in the response to pancreatic amylase. Determination of the amount of secreted carbohydrate showed that the increased secretion seen in the WT strain in response to stimulation with HPA was absent in a *cj0511* mutant (Fig. 2D). Restoration of the response to HPA in the complemented strain (Fig. 2D), shows that the defect in the *cj0511* mutant is the result of inactivation of *cj0511* and not due to a polar effect or spontaneous mutation elsewhere in the genome. To determine if Cj0511 degrades pancreatic amylase, recombinant Cj0511 (r-Cj0511) was incubated with recombinant human pancreatic amylase or with casein (Fig. 3). Partial and complete degradation of casein was apparent at 30 min and 2 h, respectively. In contrast, there was no apparent degradation of pancreatic amylase after 2 h.

277

278 **Exposure to pancreatic amylase promotes biofilm formation.** To determine if  
279 exposure to pancreatic amylase contributed to biofilm formation in *C. jejuni*, biofilms  
280 formed at the air/liquid interface in glass tubes in the presence or absence of  
281 physiological concentrations of pancreatic amylase (100 nM) were measured by  
282 crystal violet staining. With the exception of the *cj0511* mutant, all the strains showed  
283 significant increases in biofilm formation in the presence of HPA (Fig. 4A). The  
284 magnitude of the increase in biofilm in response to HPA in the *waaC*, *waaF* and  
285 *kpsM* mutants was similar to the WT (2.1-2.5-fold) demonstrating that it is  
286 independent of LOS and CPS. Although we observed increased biofilm formation by  
287 the *cj0511* mutant compared to the WT in the absence of HPA ( $p=0.003$ ), there was  
288 no significant increase in the presence of HPA in this mutant (in contrast with the  
289 complemented strain), demonstrating that a functional Cj0511 protease is required  
290 for the response (Fig. 4A). To confirm that the increased biofilm was specifically due  
291 to pancreatic amylase, the assay was repeated in the presence of 100 nM  
292 recombinant human pancreatic amylase and an increase in biofilm density of similar  
293 magnitude (2.2-fold;  $p=0.001$ ) to that obtained with the preparation of HPA was  
294 observed (Fig. 4B). Addition of a volume of chicken pancreas extract containing  
295 equivalent amylase activity also resulted in an increase in biofilm density (2.2-fold;  
296  $p<0.001$ ) (Fig. 4C).

297 Confocal laser scanning microscopy (CLSM) was used to visualise biofilms of  
298 *C. jejuni* 11168H grown in the presence or absence of HPA. In the presence of HPA  
299 there was typical biofilm structure at 48 h with adherent microcolonies of live bacteria  
300 separated by dark channels, whereas there were few adherent bacteria in the

301 absence of HPA (Fig. 4D). Examination in the x-z plane showed typical three-  
302 dimensional biofilm structure (of maximum height 160  $\mu$ m) only in the presence of  
303 HPA.

304  
305 **Pre-exposure to pancreatic amylase promotes interaction with human IECs.** To

306 determine if pre-exposure of *C. jejuni* to HPA affects the interaction with IECs,  
307 strains grown with or without 100 nM HPA were co-cultured with Caco-2 cells. *C.*  
308 *jejuni* 11168H grown in the presence of HPA showed significant increases in the  
309 number of interacting bacteria after 3, 6 and 24 h of co-culture (Fig. 5A). Growth of  
310 *C. jejuni* 81-176 in the presence of HPA also resulted in significant increases in  
311 interaction with Caco-2 cells (data not shown). In contrast, exposure of the *C. jejuni*  
312 11168H *cj0511* mutant to HPA did not result in increased interaction, whereas the  
313 complemented strain did show significant increases (Fig. 5A). Pre-exposure to HPA  
314 also resulted in increased invasion by strains 11168H (Fig. 5B) and 81-176 (data not  
315 shown). There was no increase in invasion by the *cj0511* mutant with pre-exposure  
316 to HPA whereas the complemented strain showed a significant increase (Fig. 5B).

317 To explore the effect of pre-exposure to HPA on IEC cytokine responses, we  
318 measured interleukin-8 (IL-8) secretion from T84 human colon cancer cells in  
319 response to bacteria grown with or without HPA. At 24 h, we observed a significant  
320 increase in IL-8 secretion in response to infection but growth on HPA had no effect  
321 (Fig. 5C). A similar lack of cytokine response to HPA pre-treatment was observed in  
322 THP-1 macrophages (data not shown). The effect of infection on transepithelial  
323 electrical resistance (TEER) was also investigated. No disruption in tight-junction  
324 integrity in response to *C. jejuni* grown on HPA was noted 6 h post-infection (data  
325 not shown), but there was a significant increase in translocation of the bacteria pre-

exposed to HPA compared to those grown without HPA (Fig. 5D), a difference no longer apparent at 24 h (data not shown).

### **Pre-exposure to pancreatic amylase results in increased killing of *Galleria***

***mellonella* larvae.** *C. jejuni* 11168H, 81-176 and the *cj0511* and *kpsM* mutants were grown with or without HPA, injected into ten *G. mellonella* larvae and the number of dead larvae determined at 24 h (Fig. 6). For both WT strains, a mean of 1 - 2 larvae were killed when the bacteria were grown without HPA compared to means of ~9 when grown with HPA. The increased virulence in response to HPA was lost in the *cj0511* mutant but restored in the complemented strain (Fig. 6), confirming the essential role of Cj0511 in signal detection.

### **Pre-exposure to pancreatic amylase promotes colonisation of broiler chickens.**

To determine whether pre-exposure to pancreatic amylase affects the ability of *C. jejuni* to colonise chickens, strain 11168H and the *cj0511* mutant grown with or without HPA were used to infect broiler chickens of 21 days old. At day 4, the WT strain had colonised better than the *cj0511* mutant but no difference was apparent between treatments (Fig. 7A). By day 7, exposure of the WT strain to HPA prior to infection had a significant effect ( $p=0.005$ ) (Fig. 7B). Differences in the numbers of bacteria present between infection groups were seen at day 4 ( $p=0.0006$ ) and day 7 ( $p=0.03$ ). In chickens infected with strains grown in the presence of HPA, the WT strain was present in significantly higher numbers in the ileum compared to the *cj0511* mutant at days 4 and 7 (Fig. 7C, D). Growth on HPA also significantly



increased the numbers of the WT strain in the ileum at day 7 whereas there was no such increase in the *cj0511* mutant (Fig. 7D).

### **Pre-exposure to pancreatic amylase promotes survival in stress environments.**

To assess the role of the  $\alpha$ -dextran in survival outside the host, we cultured *C. jejuni* 11168H in the presence or absence of 100 nM HPA, recovered the bacteria into either broth or PBS without HPA and determined by viable counting the numbers of surviving bacteria at room temperature and at 4°C in air. In both stress conditions, pre-exposure to HPA prolonged survival suggesting that the  $\alpha$ -dextran has a protective effect (Fig. 8).

## **DISCUSSION**

Given that *C. jejuni* has been described as a “sugary bug” (45) it is somewhat surprising that until the work described herein, no definitive identification of a polysaccharide involved in biofilm formation had been made. Indeed it has been suggested that DNA is a major component of the *C. jejuni* biofilm matrix (26). However, when *C. jejuni* is freshly isolated from animal or human stools it is highly mucoid (Allan, unpublished observation), suggesting that a polysaccharide matrix is being produced. This led us to hypothesize that a host factor was responsible for inducing the polysaccharide EPM. Such interkingdom signalling between eukaryotes and prokaryotes was first identified over a century ago (46). Over the last decade or so it has been established that interkingdom signalling between bacteria and hosts, via “hormones” is widespread (47). One of the first reports of this was the effect of adrenaline and noradrenaline on *E. coli* (48). There are a limited number of reports

on host cell molecules affecting biofilm formation by bacteria. For example, bile salts enhance biofilm formation by *C. jejuni* (26), *Listeria monocytogenes* (49) and *Vibrio cholerae* (50). In the case of *Streptococcus pneumoniae*, a number of host factors affect biofilm formation and structure including norepinephrine (51), and extracytoplasmic ATP (52). Our work shows for the first time that avian and mammalian pancreatic amylase can act as an interkingdom signalling molecule and enhance *C. jejuni* growth and biofilm formation. It is important to emphasise that a mucoid colony is produced on Brucella agar in response to pancreatic amylase. This medium does not contain starch, indicating that the response is directly to the presence of amylase protein and is not dependent on amylase starch degrading activity. This was confirmed in the biofilm assay which was also carried out in medium lacking starch. To our knowledge this is only the second example of a proteinaceous host component that can enhance bacterial biofilm formation, the first being the cytokine interleukin 1 $\beta$  which enhances biofilm formation by *Staphylococcus aureus* (53). Interestingly, *C. rectus* did not appear to detect pancreatic amylase suggesting that the response may be a specific adaptation to host environments. It will be interesting to investigate in future work whether the enteric and oral *Campylobacter* spp. are able to detect the presence of salivary amylase, given its similarity in sequence and structure to pancreatic amylase.

The polysaccharide secreted by *C. jejuni* was definitively identified as  $\alpha$ -dextran and the ease with which it was removed from the bacterial cell surface, by simply washing in saline, indicates that it is a loosely-associated 'slime polysaccharide' as oppose to the capsular polysaccharides that are covalently attached to the cell wall (16, 54). Given that *C. jejuni* is capable of producing complex glycan structures (18),

the production of a simple  $\alpha$ -dextran is intriguing. Dextrans are produced by oral streptococci and *Leuconostoc* spp. from sucrose using dextransucrases, cell wall-anchored enzymes of the glycoside hydrolase superfamily (56). Searching the translated *C. jejuni* genome sequences with known dextransucrase sequences did not identify homologues, suggesting that dextran synthesis in *C. jejuni* occurs by a novel mechanism. The source of the glucose monomer, whether it is taken up from the environment or synthesized from amino acids, awaits experimental investigation.

The fact that HPA enhances the growth of *C. jejuni* suggested that it may be used as a nutrient source and Cj0511, predicted as a serine protease of the C-terminal protease (CTP) family and known to be secreted in OMVs (43), seemed a likely candidate for degrading pancreatic amylase. Our data shows that a *cj0511* mutant did not display the response to HPA, a phenotype that was restored in a complemented strain, confirming the role of Cj0511 specifically. However, there was no apparent change in migration of recombinant human amylase on an SDS-PAGE gel following incubation with Cj0511 even though partial casein degradation was apparent in 30 min and complete digestion in 2 h. The recombinant amylase is tagged with 6 histidine residues at the C-terminus and C-terminal proteolysis of even a single amino acid would result in a reduction of ~1 kDa in the molecular weight of the protein which would be apparent on a 16% polyacrylamide gel. In this context, it is important to emphasise that the polyhistidine-tagged human recombinant amylase preparation was active, since we showed it induced biofilm formation in the wild-type strain. Thus, interference by the polyhistidine tag cannot explain why we fail to see cleavage by r-Cj0511 *in vitro*. Since Cj0511 is N-glycosylated in *C. jejuni* (Young et

al., 2002), an absence of glycosylation in r-Cj0511 is another plausible explanation. However, we have shown that a *pglB* mutant (i.e. defective for N-glycosylation; 55) still responds to pancreatic amylase (Jowiya and Allan, unpublished), which discounts this explanation also. Thus we conclude that Cj0511 does not digest pancreatic amylase, at least *in vitro*. Bacterial CTPs are involved in a range of physiological processes. The *Escherichia coli* tail-specific protease processes penicillin binding proteins and regulates cell morphology (57). CTPs with roles in virulence have also been described: for example, CtpA of *Brucella suis* is required for virulence in a mouse infection model (58). A recent study by Karlyshev and co-workers showed that a *cj0511* mutant was severely attenuated in its ability to colonise chickens (42). Since Cj0511 does not appear to degrade pancreatic amylase, we conclude that it is involved at a later stage in the molecular mechanism. Elucidation of its precise role in the response to pancreatic amylase requires further research.

Having established that pancreatic amylase induces secretion of an  $\alpha$ -dextran, we next addressed the biological consequence. Growth on physiological concentrations of HPA resulted in increased biofilm formation in two different WT strains. Moreover, the increase in biofilm formation induced by HPA was dependent on Cj0511. Since the purity of the commercial HPA preparation is not guaranteed, we tested purified recombinant human pancreatic amylase in the biofilm assay and observed an increase in biofilm formation of similar magnitude to that induced by HPA, confirming that the response is to pancreatic amylase specifically. Biofilm formation was also increased in response to an extract of chicken pancreas suggesting that the  $\alpha$ -

dextran promotes biofilm formation in the intestines of both mammalian and avian hosts.

The study next investigated the effect of HPA exposure on the interaction of *C. jejuni* with IECs. Growth on HPA resulted in a significant increase in adhesion and invasion of *C. jejuni* in Caco-2 cells. As the neutrophil chemoattractant IL-8 has a role in the response to *C. jejuni* (59), we studied production by T84 cells as they are more potent cytokine responders than Caco-2 cells (60). Surprisingly, no difference in IL-8 secretion in response to bacteria grown with or without HPA was observed. Pre-exposure to HPA led to a significant increase in bacterial translocation 6 h post-infection, however, without loss of tight junction integrity. Collectively, these data suggest that the  $\alpha$ -dextran modulates early host-pathogen interactions by promoting rapid intracellular transfer while avoiding overt host inflammatory responses. It is intriguing to speculate that an  $\alpha$ -1,6-dextran evolved as a colonisation factor since it exhibits sufficient similarity to dietary starch to be tolerated by the immune system yet is resistant to the  $\alpha$ -1,4-glucan glucanohydrolase activity of pancreatic amylase.

In the *G. mellonella* infection model, growth of *C. jejuni* on pancreatic amylase resulted in increased virulence and this is again dependent on Cj0511. *G. mellonella* larvae have been used as a model to study infection by *C. jejuni* (36) and other enteric pathogens as they possess both humoral and cellular immune systems (61). The increased insecticidal activity of *C. jejuni* grown on HPA suggests that biofilm formation may contribute to resistance to these immune mechanisms. It is notable that the insecticidal activity observed for *C. jejuni* 11168H and 81-176 in the absence of pancreatic amylase was lower than that reported by Champion *et al.* (36) for these strains. We assume that the decreased virulence observed in our study is a

reflection of culture on Mueller Hinton agar as oppose to Columbia blood agar which was used by Champion *et al.*

In the laboratory, *C. jejuni* is sensitive to environmental stresses such as drying, chilling, and exposure to atmospheric oxygen yet the bacterium exhibits a remarkable ability to persist in food processing facilities and natural environments (62). We have shown that pre-exposure of *C. jejuni* to pancreatic amylase prolongs survival in atmospheric conditions at ambient and refrigeration temperatures. This demonstrates that the  $\alpha$ -dextran, which will be expressed by *C. jejuni* expelled into the environment from a host organism, is stable and may be important for the environmental resilience of the bacterium.

In conclusion, we propose a new model in which *C. jejuni* uses host pancreatic amylase, encountered as it passes the pancreatic duct, as a signal for secretion of an  $\alpha$ -dextran which promotes biofilm formation and contributes to colonisation of the mammalian and avian intestine and increases resistance to host immune functions. We have shown that Cj0511, a secreted serine protease, is required for the response to pancreatic amylase although its precise function in the response is currently unknown. The demonstration of a secreted dextran that leads to biofilm formation is an important finding that is expected to enable the design of intervention strategies to reduce the burden of *C. jejuni* in the food chain and ultimately to reduce the incidence of human enteric disease.

## ACKNOWLEDGMENTS

We are grateful to Duncan Gaskin and Arnoud van Vliet for the gift of pC46fdxA. We thank Nicky Mordan for help with confocal microscopy, David Boniface for help with statistical analyses and Peter Mullany for reading the manuscript.

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Bacterial strain	Characteristics	Reference
<i>C. jejuni</i> 11168H	Hyper-motile variant of NCTC 11168; good coloniser of chickens	(63)
<i>C. jejuni</i> 81-176	Clinical isolate from human diarrhoea sample	(64)
<i>C. jejuni</i> 81116	Waterborne human outbreak strain	(65)
<i>C. jejuni</i> X	Clinical isolate from patient with enteritis	(66)
<i>C. jejuni</i> G1	Clinical isolate from patient with Guillain-Barré syndrome	(67)
<i>C. jejuni</i> 11168H <i>kpsM::aphA3</i>	Mutant in gene encoding ABC transporter involved in capsule assembly; no CPS expression	(68)
<i>C. jejuni</i> 11168H <i>waaC::aphA3</i>	Mutant in gene encoding heptosyltransferase I; expresses truncated LOS	<i>Campylobacter</i> Resource Facility ( <a href="http://crf.lshtm.ac.uk/index.htm">http://crf.lshtm.ac.uk/index.htm</a> )
<i>C. jejuni</i> 11168H <i>waaF::aphA3</i>	Mutant in gene encoding heptosyltransferase II; expresses truncated core oligosaccharide	<i>Campylobacter</i> Resource Facility ( <a href="http://crf.lshtm.ac.uk/index.htm">http://crf.lshtm.ac.uk/index.htm</a> )
<i>C. jejuni</i> 11168H <i>cj0511::aphA3</i>	Mutant in gene encoding secreted carboxyl-terminal protease.	<i>Campylobacter</i> Resource Facility ( <a href="http://crf.lshtm.ac.uk/index.htm">http://crf.lshtm.ac.uk/index.htm</a> )
11168H <i>cj0511::aphA3</i> pWJ4	Complemented strain containing <i>cj0511</i> gene and Cam <sup>r</sup> inserted into <i>cj0046</i>	This study
<i>C. rectus</i> NCTC 11489	Type strain, from human periodontal pocket	Public Health England culture collections

<i>E. coli</i> JM109	Cloning host	Promega
<i>E. coli</i> BL21 (DE3)	Expression host	New England Biolabs
<b>Plasmid</b>		
pJMK30	pUC19 carrying <i>C. coli</i> Kan <sup>r</sup> gene <i>aphA-3</i>	(69)
pGEM-T-Easy	Cloning vector enables TA cloning	Promega
pC46fdxA	Complementation vector containing <i>fdxA</i> promoter, Cam <sup>r</sup> selection marker.	(34)

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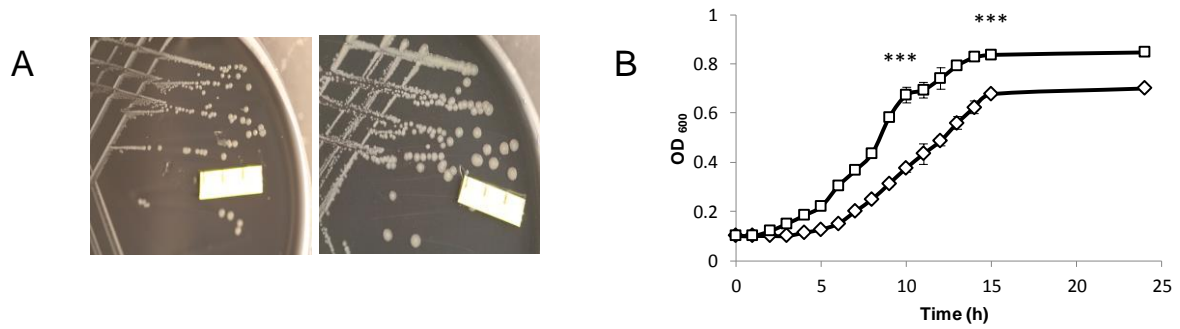
726 Table 2. Oligonucleotides

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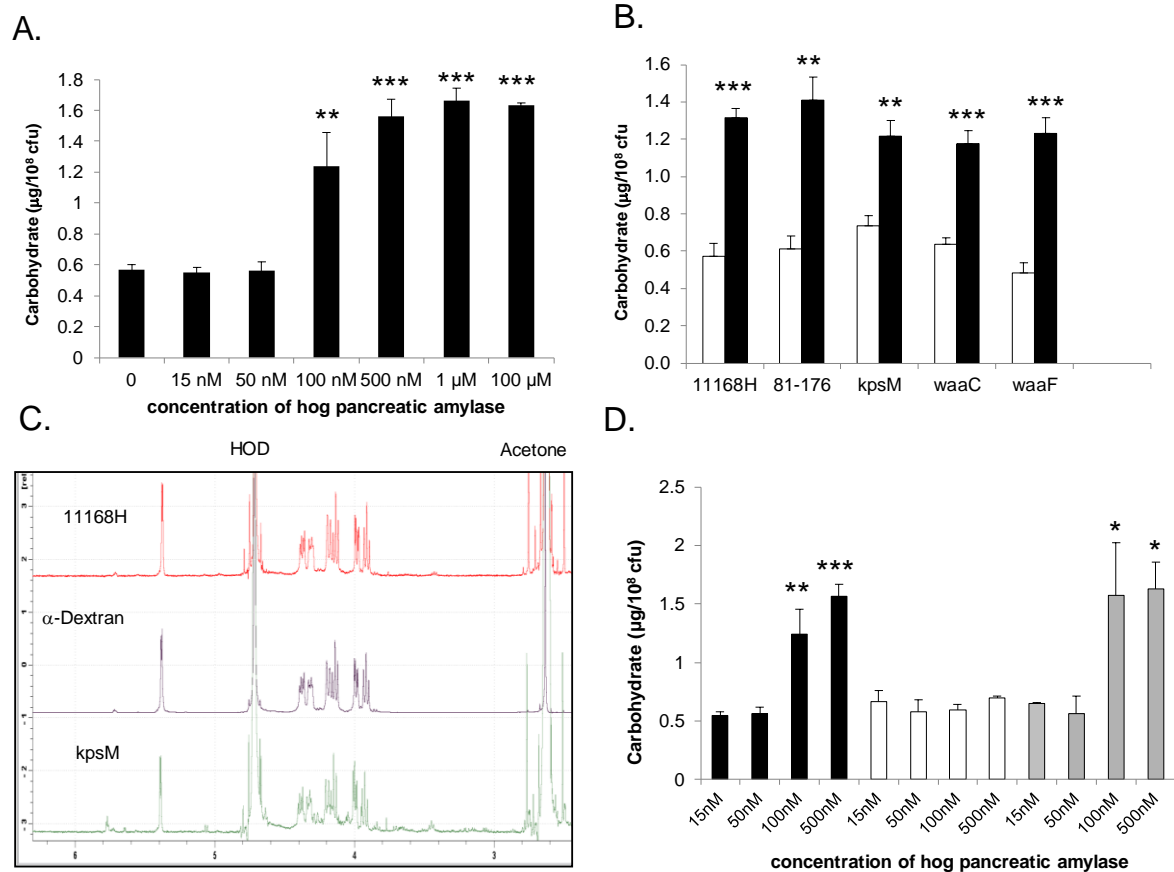
Name	Sequence (5'-3')	Use
WJ10F	GT <u>CGTCTCACATG</u> TTGAAAACAAAACG	Amplification of <i>cj0511</i> for cloning in pC46fdxA (BsmBI site underlined)
WJ10R	GT <u>CGTCTCACATG</u> TTATTGTCCTTGTTG	Amplification of <i>cj0511</i> for cloning in pC46fdxA (BsmBI site underlined)
WJ4	GTAAATTTTTGATTATCAAAATTACATTATTTAAG	Orientation of <i>cj0511</i> with respect to $P_{fdxA}$ in pC46fdxA; anneals in $P_{fdxA}$
WJ11	GGAACACAGCAGAGCACTTG	Confirmation of <i>cj0511</i> complementing strain; anneals in <i>cjs03</i> RNA coding sequence located 5' to <i>cj0046</i>
WJ12	CCTGGAGAAGTATTAGATAGTAGCG	Confirmation of <i>cj0511</i> complementing strain; anneals in <i>cj0053c</i> located 3' to <i>cj0046</i>

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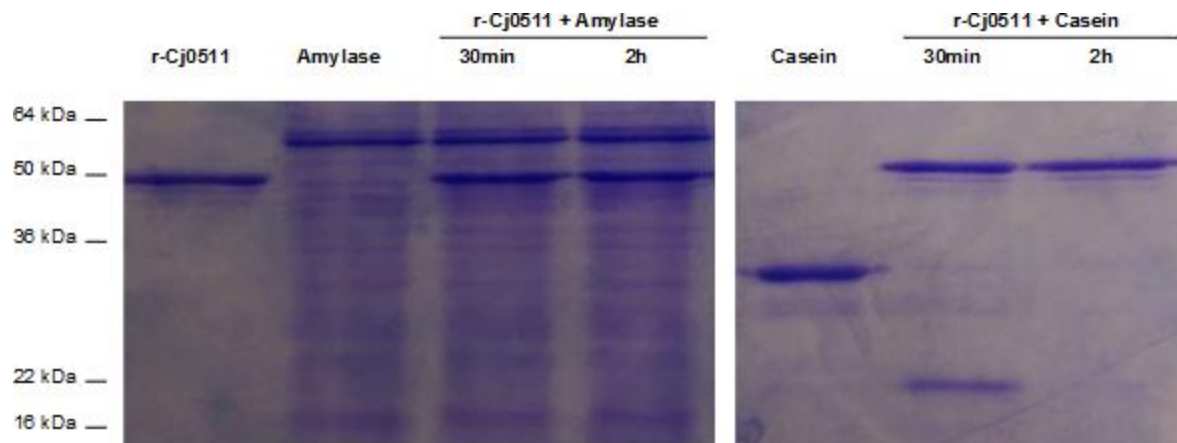


**FIG. 1.** Exposure to pancreatic amylase promotes growth and results in a large, mucoid colony. A. *C. jejuni* 11168H grown at 37°C for 72 h on BA containing 0.4% charcoal without (left) or with (right) 100  $\mu$ M HPA. The ruler marks 5 mm intervals. B. *C. jejuni* 11168H growth with shaking (50 rpm) in 5% CO<sub>2</sub> at 37°C in MEM- $\alpha$  with (squares) or without (diamonds) 100 nM HPA. Data reported as means and standard deviations (SD) from three independent experiments. The statistical significance of the difference in growth in the presence and absence of HPA was assessed at two time points (10 and 15 h) using a two-sample t-test, assuming unequal variance; \*\*\* $p$ <0.001.

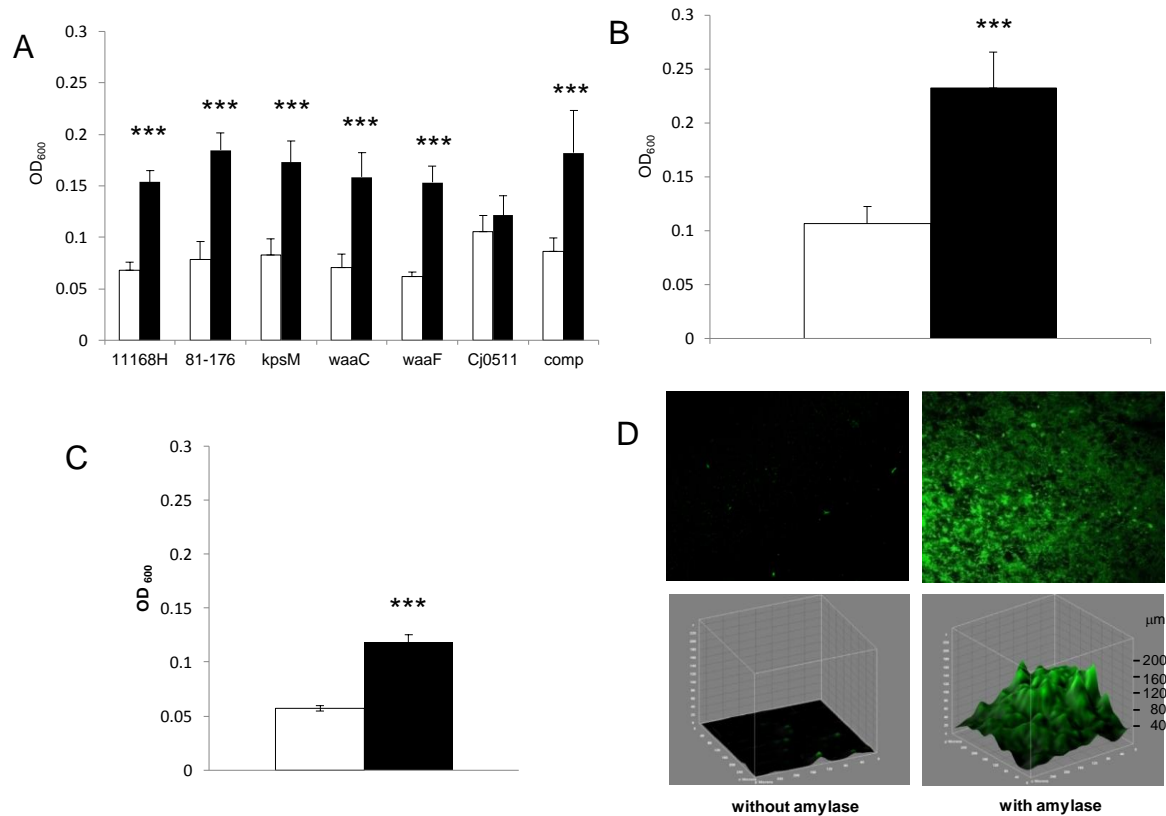


**FIG. 2. Physiological concentrations of pancreatic amylase induces  $\alpha$ -dextran secretion in *C. jejuni* and the secreted protease, Cj0511 is required.** (A) Total extracellular carbohydrate in *C. jejuni* 11168H grown in the presence of increasing concentrations of HPA. (B) Total extracellular carbohydrate in different *C. jejuni* strains grown in the presence (black bars) or absence (white bars) of 100 nM HPA. The *kpsM*, *waaC* and *waaF* mutants were created in strain 11168H. (C)  $^1\text{H}$ -NMR spectra of extracellular material from *C. jejuni* 11168H, a *kpsM* mutant and a commercial  $\alpha$ -dextran recorded in  $\text{D}_2\text{O}$  at  $7^\circ\text{C}$  using acetone as an internal standard on a Bruker Avance 400 MHz spectrometer. (D) Total extracellular carbohydrate in *C. jejuni* 11168H (black bars), a *Cj0511* mutant (white bars) and a *Cj0511* complemented strain (grey bars) grown on MHA with increasing concentrations of

HPA. For (a), (b) and (d), data reported as mean  $\pm$  SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of HPA; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .



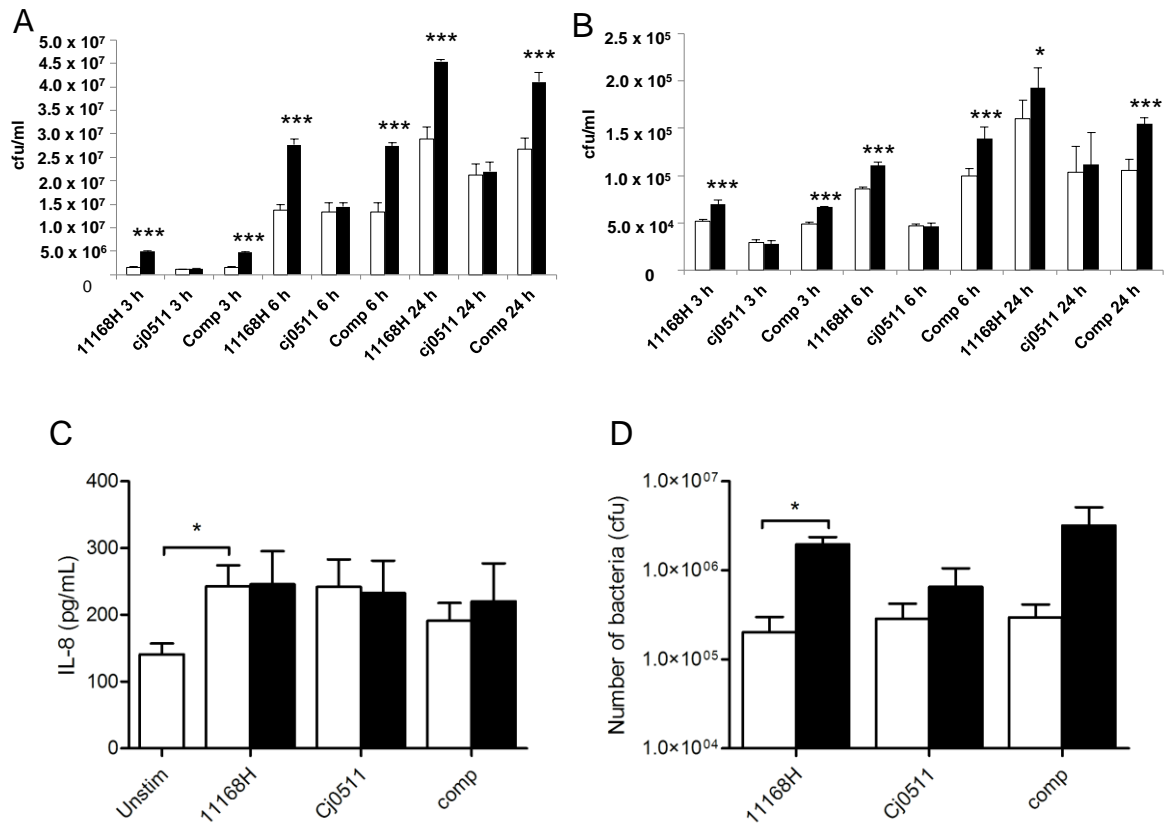
**Fig. 3. Proteolysis of casein and pancreatic amylase by r-Cj0511.** Coomassie-stained SDS-polyacrylamide gel showing no detectable proteolysis of polyhistidine tagged human pancreatic amylase. Casein served as a positive control.



**Fig. 4. Exposure to pancreatic amylase promotes biofilm formation.** (A) Biofilm formation at 48 h by *C. jejuni* strains on glass tubes in the presence (black bars) or absence (white bars) of 100 nM HPA. Data reported as means and SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of HPA; \*\*\* $p < 0.0001$ . (B) Biofilm formation at 48 h by *C. jejuni* 11168H on glass tubes in the presence (black bars) or absence (white bars) of 100 nM recombinant human amylase. Data reported as mean  $\pm$  SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of pancreatic amylase; \*\*\* $p < 0.001$ . (C) Biofilm formation at 48 h by *C. jejuni* 11168H on glass tubes in the presence (black bars) or absence (white bars) of chicken pancreas extract. Data reported as mean  $\pm$  SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of pancreatic

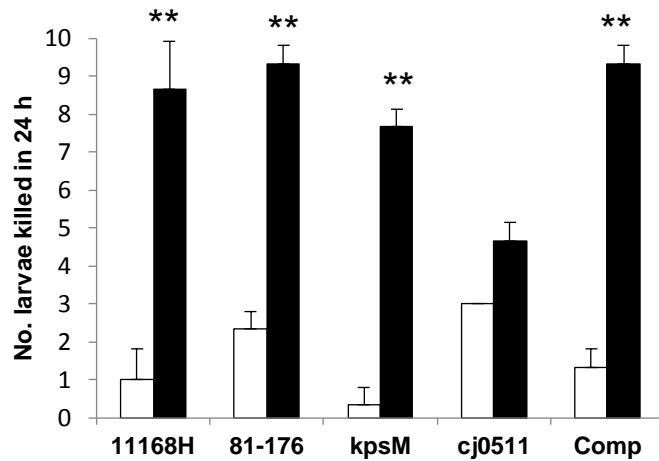


amylase; \*\*\* $p < 0.001$ . (D) CLSM of Live/Dead stained *C. jejuni* 11168H biofilm at 48 h. Top panels, confocal images of bacteria in x-y plane, bottom panels, digital images in x-z plane.



**FIG. 5. Pre-exposure to pancreatic amylase promotes interaction with human intestinal epithelial cells.** (A) Interaction with Caco-2 cells by *C. jejuni* 11168H grown in the presence (black bars) or absence (white bars) of 100 nM HPA. Bacteria were co-cultured with Caco-2 cells for 3, 6 and 24 h. (B) Invasion of Caco-2 cells by *C. jejuni* 11168H grown in the presence (black bars) or absence (white bars) of 100 nM HPA. (C) Apical IL-8 levels in T84 cells at 24 h in response to *C. jejuni* 11168H, the *Cj0511* mutant and the complemented strain grown in the presence (black bars) and absence (white bars) of 100 nM HPA. (D) Translocation of *C. jejuni* in T84 cells at 6 h. *C. jejuni* 11168H, a *Cj0511* mutant and a complemented strain were grown in the presence (black bars) and absence (white bars) of 100 nM HPA. For (A) to (C), data reported as mean  $\pm$  SD from three independent experiments. For (D), data reported as mean  $\pm$  SD from three independent experiments for *C. jejuni* 11168H

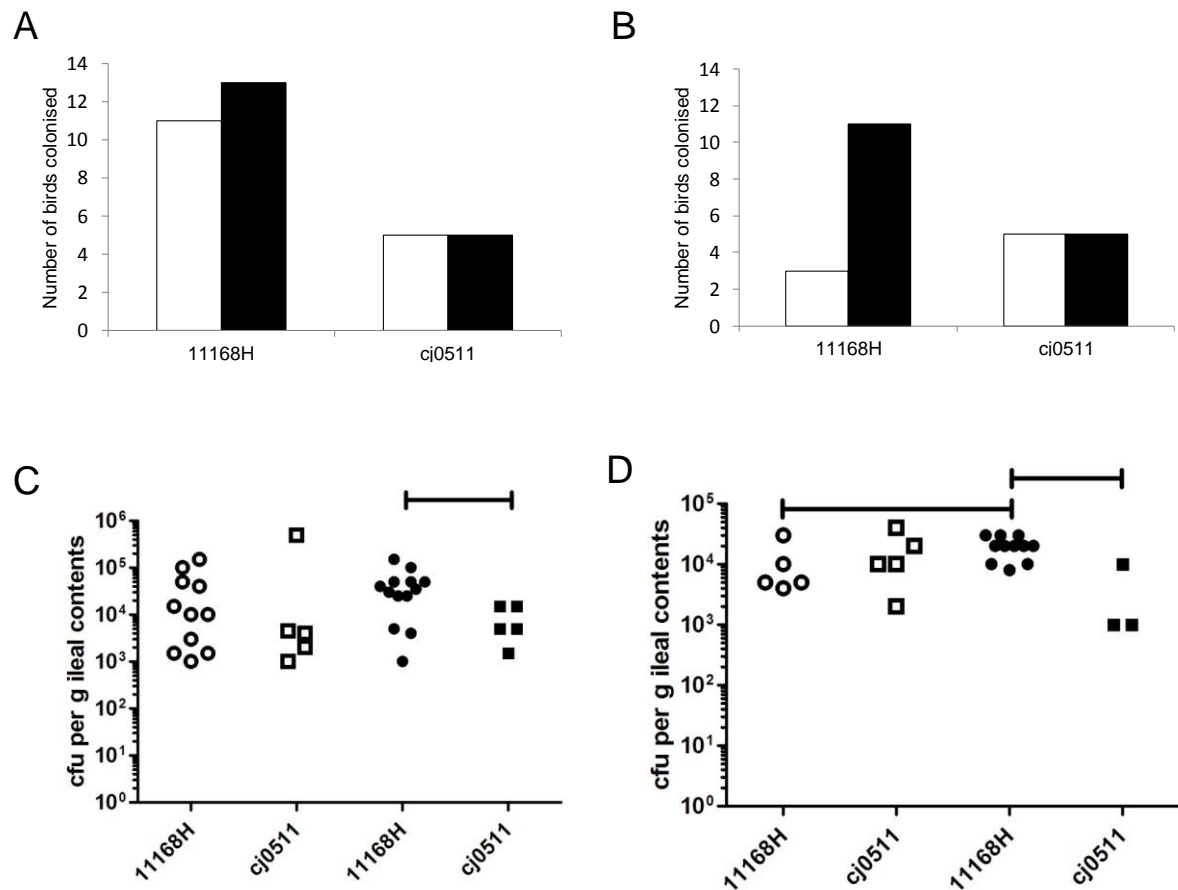
and the *cj0511* mutant. For the *cj0511* complemented strain, the mean +/- SD from two independent experiments is reported as a result of contamination in the third experiment, thus no statistical analysis is presented. \*\*\* $p < 0.0001$ , \* $p < 0.05$ .



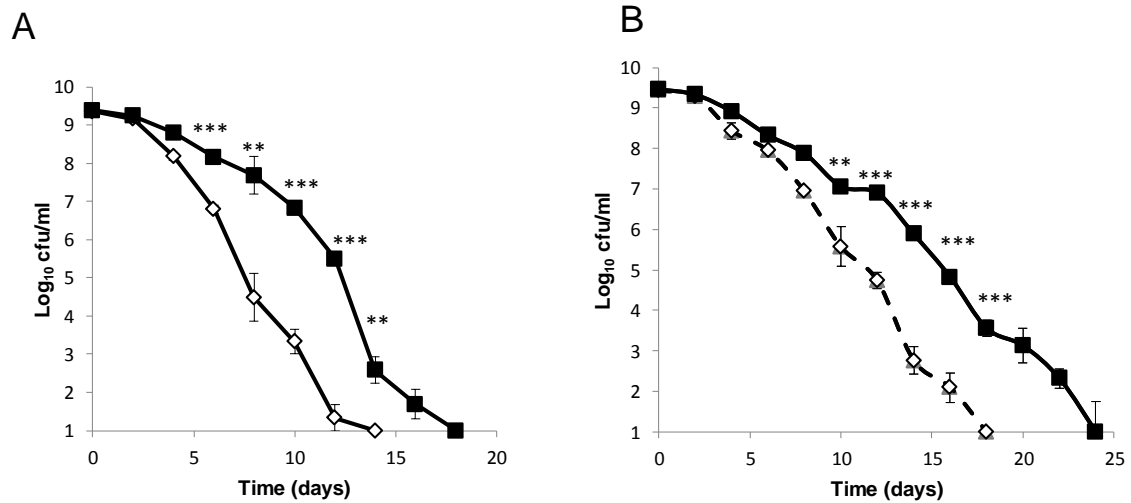
**FIG. 6. Pre-exposure to pancreatic amylase promotes infection of *Galleria mellonella* larvae.** Killing of *G. mellonella* larvae by *C. jejuni* grown in the presence (black bars) or absence (white bars) of 100 nM HPA. 10 larvae were infected with each strain or with PBS alone. Injection with PBS did not kill any larvae. The *kpsM* mutant and the *cj0511* mutant and complemented strain were derived from *C. jejuni* 11168H. Data reported as mean and SD from three independent experiments.

Asterisks indicate statistically significant increases in the presence of HPA;

\*\*p=0.001.



**FIG. 7. Pre-exposure to pancreatic amylase promotes colonisation of broiler chickens.** (A) and (B) number of birds from which *Campylobacter* was isolated from the ileum at 4 d and 7 d post infection, respectively. *C. jejuni* was grown with (black bars) or without HPA (white bars). n=15 birds per group. By day 7, exposure of the WT strain to HPA prior to infection had a significant effect (p=0.005). (C) and (D) cfu of *Campylobacter* per gram of ileal contents in birds 4 d and 7d post infection, respectively. Open symbols show strains grown without HPA, closed symbols show strains grown with HPA. Bar indicates statistically significant difference. n=15 birds per group.



**FIG. 8. Pre-exposure to pancreatic amylase promotes survival in stress environments.** (A). Survival at 4°C of *C. jejuni* 11168H grown with (squares) or without (diamonds) HPA. (B). Survival at 20°C in air of *C. jejuni* 11168H grown with (squares) or without (diamonds) HPA. Data reported as means  $\pm$  SD from three independent experiments. Statistical significance between the counts in the presence and absence of HPA was assessed using a two-sample t-test, assuming unequal variance; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .